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Molecular Genetics of Heterokaryon Incompatibility in Filamentous Ascomycetes

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INTRODUCTION

What Is Heterokaryon Incompatibility?

In filamentous fungi, both sexual and vegetative recognition systems exist. Sexual recognition is controlled by the mating type locus (for a review, see reference 22), whereas vegetative recognition is controlled by vegetative (or heterokaryon) incompatibility systems. Vegetative self/nonself recognition appears to be critical in these organisms because they spontaneously undergo vegetative cell fusion events within but also between individuals. These cell fusions (anastomoses) lead to cytoplasmic mixing and to the formation of vegetative heterokaryons (i.e., cells containing different nuclear types). The viability of these heterokaryons is genetically controlled by specific loci termed *het* (for heterokaryon incompatibility) or *vic* (for vegetative incompatibility) loci. A *het* locus can be defined as a locus at which heteroallelism cannot be tolerated in a heterokaryon. When two fungal individuals that differ genetically at a *het* locus fuse, the resulting heterokaryotic cells are rapidly destroyed or severely inhibited in their growth, depending on the *het* locus that is involved (9, 39, 73). (Fig. 1).

Genetic dissection of vegetative incompatibility systems in ascomycetes has only been undertaken in a limited number of species, including *Neurospora crassa* (39, 61, 71), *Podospora anserina* (12, 73), *Aspergillus nidulans* (4), and *Cryphonectria parasitica* (23). The number of *het* loci varies from one species to another (from 6 to 11 in the species mentioned above). In most heterokaryon incompatibility systems, the incompatible genes are alleles, but nonallelic systems in which incompatibility is triggered by the interaction of two genes at distinct loci have also been described (12). For numerous additional species in which the different *het* loci have not yet been identified, vegetative compatibility groups (VCGs) have been defined. Classification of strains in VCGs can be a convenient tool for analyzing fungal populations (for an example, see reference 3). In most cases in natural populations, the number of VCGs is considerable. In practice, a confrontation between wild-type isolates from the same or different populations most often results in an incompatible interaction (12, 59, 62).

The Question of Biological Significance

Heterokaryon incompatibility is a widespread phenomenon among filamentous fungi, but its biological significance remains a puzzle. Why do these genetic systems exist? What is their biological function? Do they actually have any? Two contrasting views exist on the subject. First, it has been proposed that heterokaryon incompatibility genes exist to limit heterokaryon formation between unlike individuals. In that hypothesis, *het* genes constitute bona fide self/nonself recognition systems. What could be the benefit of limiting heterokaryosis? It has been proposed that genetic control of heterokaryosis might limit the horizontal transfer of infectious cytoplasmic elements such as senescence plasmids, mycoviruses, transposons, and debilitated organelles (19). *het* genotype differences have indeed been shown to limit to some extent the transfer of such cytoplasmic replicons (2, 19, 21, 28, 50, 93). Control of heterokaryosis might also prevent different forms of nuclear parasitism, for instance, exploitation of an individual by unadapted nuclei that possess a fitness advantage in the heterokaryon (46) or resource plundering by conidia landing on an established maternal culture of a distinct individual (27). In these hypotheses, the function of *het* genes is to preserve genetic individuality.

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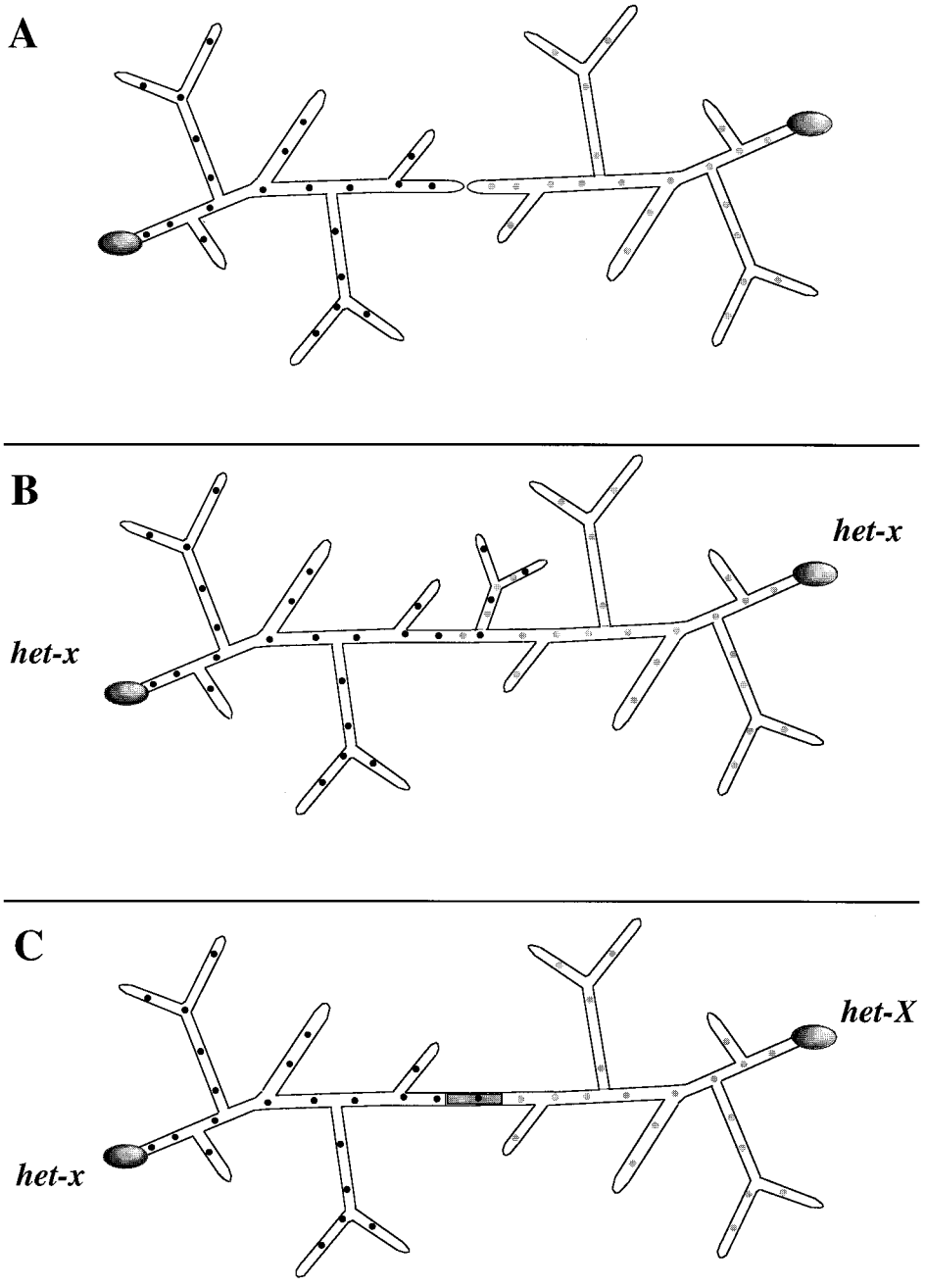

FIG. 1. Schematic representation of heterokaryon incompatibility. (A) When two different fungal individuals meet, they spontaneously undergo a cell fusion event or anastomosis. (B) If the two individuals have the same *het* genotype, a heterokaryon is established. (C) If the two strains differ in *het* genotype, the heterokaryotic cells are destroyed or severely inhibited in their growth.

The alternative view proposes that heterokaryon incompatibility does not have any function in natural populations but simply constitutes a sort of evolutionary accident. In a given population, a number of selectively neutral polymorphisms exist. Among these polymorphisms, a fraction would be detrimental at the heteroallelic state, when the two variants are reunited in the same cytoplasm in a heterokaryon. This class of polymorphism would then define the *het* genes. In this hypothesis, this phenomenon is characteristic of filamentous fungi simply because they are practically the only organisms capable of natural heterokaryosis. In other phyla, *het*-like genes (genes that are deleterious at the heteroallelic state) should exist and could be detected as genes leading to hybrid inviability or sterility in diploids (26).

These two contrasting hypotheses (which can be caricatured as the accident and the allorecognition hypotheses) imply a number of specific properties for the *het* genes. The accident hypothesis postulates that *het* genes do not exist to limit heterokaryosis and should therefore have other functions in fungal biology. The allorecognition hypothesis does not imply the

TABLE 1. Cloned *het* genes of *N. crassa* and *P. anserina*

Species and gene	No. of alleles	Size of encoded polypeptide (amino acids)	Protein feature	Reference
N. crassa				
mat A-1		293	α-Box	40
mat a-1		381	HMG box	88
het-C	3	966	Signal peptide, glycine-rich repeats	79
het-6	2	680	Similarity to TOL and HET-E	85
un-24	2	929	Ribonucleotide reductase	84
P. anserina				
het-s	3	289	Prion protein	24
het-c	4	208	Similarity to GLTPs	77
het-e	4	1,056	WD-40 repeats, GTP-binding site	81

opposite. Genes with cellular functions might have been recruited to perform self/nonself recognition. Therefore, the identification of a cellular function associated with a *het* gene strengthens the accident hypothesis but does not rule out the alternative. It simply rejects a model in which *het* gene function is strictly limited to self/nonself recognition.

The second and main prediction is that polymorphism will be selected for in the allorecognition hypothesis and generated under neutrality in the accident hypothesis. Positive Darwinian selection is a common feature of various recognition and host defense systems. Discrimination between the two hypotheses should be possible by analyzing variability in *het* genes in populations to search for hallmarks of positive selection.

SCOPE OF THE REVIEW

This review focuses on the molecular genetics of heterokaryon incompatibility in *P. anserina* and *N. crassa*, the two species in which *het* genes have been isolated. Molecular characterization of *het* genes is described as well as work on suppressor genes and on genes induced during the incompatibility reaction in *P. anserina*. It is by no means a comprehensive description of heterokaryon incompatibility. Work on the genetics of incompatibility in other ascomycetes will only be mentioned marginally. These aspects have been discussed more thoroughly in previous reviews (8, 41, 53).

HETEROKARYON INCOMPATIBILITY IN *NEUROSPORA CRASSA*

At least 11 *het* loci exist in *N. crassa*. Five of them, the mating type locus and *het-c*, *-d*, *-e*, and *-i*, were originally identified using forced heterokaryons between nearly isogenic strains (39, 71). The *het-I*/*het-i* interaction does not lead to an immediate destruction of the heterokaryotic cell, as the other systems do, but to the loss of one nuclear component of the heterokaryon. The other *het* loci, *het-5* through *het-10*, were characterized using tester translocation strains containing duplication-generating chromosome rearrangements (61). When these translocation strains are crossed with the wild type, a predictable proportion of the progeny contain a duplication of the translocated chromosome segment. If a *het* locus is present in the translocated chromosome segment and if the parent strains bear different alleles at that locus, growth of the partial diploids will be inhibited (68). These translocation strains have been used to analyze *het* gene constitution in natural isolates because they dispense with the construction of the nearly isogenic strains required for *het* genotyping in forced heterokaryons.

All *N. crassa het* loci behave genetically as allelic systems. Among them, three (the mating type locus, *het-c*, and *het-6*) have been characterized at the molecular level (40, 79, 84, 85, 88)) (Table 1). A specific suppressor of mating-type-associated incompatibility (the *tol* gene, for tolerant) has also been isolated (83) (Table 2). *het-c*, *het-6*, and *tol* have all been cloned using similar approaches that combine positional cloning and a transformation assay involving induction of the incompatibility reaction.

Mating Type-Associated Incompatibility and Its Suppressor *tol*

In *N. crassa*, the mating type is bipolar. It exists as two alleles, termed *a* and *A*. Fertilization is mediated by mating type-specific pheromones and only occurs between gametes of opposite mating types. After cytogamy, the male nucleus is transported to the ascogonium. The two haploid nuclei undergo synchronous divisions, and then the mating type locus specifies nuclear identity to ensure that two nuclei of opposite

TABLE 2. Suppressors of heterokaryon incompatibility and induced genes

Type	Gene	Connection with incompatibility	Size of encoded polypeptide (amino acids)	Protein features	Reference
Suppressors	*N. crassa tol*	Suppressor of mating type-associated incompatibility	1,011	Leucine-rich repeats	83
	P. anserina				
	mod-A	Suppressor of nonallelic incompatibility	687	SH3-binding domain	5
	mod-D	Partial suppressor of *het-C het-E*	354	Gα subunit	56
	mod-E	Partial suppressor of *het-R het-V*	701	HSP90	57
Induced genes	*idi-1*	Induced by nonallelic incompatibility	201	Signal peptide	18
	idi-2	Induced in *het-R het-V*	157	Signal peptide, cysteine rich	
	idi-3	Induced by nonallelic incompatibility	196	Signal peptide	
	pspA	Induced by nonallelic incompatibility	532	Serine protease	

mating types enter the ascogenous hyphae in which eventually caryogamy and meiosis occur. In *N. crassa*, in addition to its role in the sexual cycle, the mating type locus also functions as a heterokaryon incompatibility locus. Vegetative coexpression of opposite mating types is lethal (6, 38). Therefore, although cytogamy between opposite mating types is required for entry into the sexual cycle, the *a* and *A* alleles cannot coexist in the vegetative state. The *a* and *A* mating type alleles have been cloned, and they are termed idiomorphs, as they differ completely in sequence (43). Three distinct genes have been identified in the *A* idiomorph. *mat A-1* specifies mating identity and is required for postfertilization functions, whereas *mat A-2* and *mat A-3* are only required after fertilization (34, 35, 40, 42). The *a* idiomorph encodes a single polypeptide, MAT a-1, that specifies all functions of the *a* mating type (88). The vegetative incompatibility function is carried by the *mat A-1* and *mat a-1* genes. Both encode putative transcriptional regulators containing potential DNA binding domains. The MAT a-1 protein displays an HMG box, and MAT A-1 contains an α-box (a domain conserved in various mating type polypeptides, including MATα1 of *Saccharomyces cerevisiae*).

The selection of mutations that restore growth to an incompatible strain is a general approach that has been widely used to dissect heterokaryon incompatibility. Using this approach, a number of mutations in *mat a-1* or *mat A-1* that restore the growth of a mixed mating type heterokaryon have been isolated (44, 45, 80). Mutants which lost the incompatibility function but retained the mating function have been isolated. For instance, an arginine-to-serine substitution at amino acid position 258 in the *mat a-1* product inactivates only the incompatibility function (45, 88). In vitro, the DNA-binding activity of this *mat a-1* mutant is unaffected. Similarly, deletion within the HMG domain alleviates DNA binding but does not affect incompatibility function (70). The MAT A-1 and MAT a-1 polypeptides interact in the yeast two-hybrid system, and mutations that abolish this interaction affect incompatibility but not mating (T. C. Badgett and C. Staben, unpublished results). These data suggest that these polypeptides mediate incompatibility and mating identity functions during the sexual cycle by distinct mechanisms. It appears that the incompatibility function is not mediated by a transcriptional regulation activity but relies on direct interaction of the two polypeptides.

The fact that the sexual and vegetative functions of the mating type locus are distinct is also illustrated by the fact that the *tol* mutations, which suppress mating type-associated incompatibility, do not affect mating. The *tol* mutations are recessive and allow mixed mating type heterokaryons or partial diploids to grow vigorously but do not suppress incompatibility triggered by other *het* loci (54, 64). The *tol* gene has recently been isolated by chromosome walking from the tightly linked *trp-4* marker (83). A *tol*-containing cosmid has been identified by locating crossover points between *tol* and flanking markers along a cosmid contig spanning the region. A functional approach based on complementation of the *tol* mutation was used to characterize the *tol* open reading frame (ORF). *tol* encodes a 1,011-amino-acid-long polypeptide. It possesses a putative leucine-rich repeat motif, a motif involved in protein-protein interactions. *tol* mutants inactivated by repeat-induced point mutations do not display any particular phenotype in addition to the suppression of the mating type-associated incompatibility. The *a* and *A* mating type genes do not control *tol* expression, which suggests that the model in which *tol* is viewed as a "death gene" transcriptionally induced in the mixed mating type heterokaryon has to be ruled out. *tol* expression is turned off during the sexual cycle, which readily explains why the incompatibility reaction does not occur during the sexual cycle (83).

Mating type-associated incompatibility is not found in all filamentous ascomycetes, nor is it characteristic of the *Neurospora* genus. For obvious reasons, pseudohomothallic species like *P. anserina* and *Neurospora tetrasperma* do not display mating type-associated incompatibility, but numerous other heterothallic species, *Neurospora sitophila* for instance, are devoid of mating type-associated incompatibility (69). This phenomenon is not unique to *N. crassa*; it is found in unrelated species such as *Ascobolus stercorarius*, *Aspergillus heterothallicus*, and *Sordaria brevicollis* (cited in reference 83). The emergence or disappearance of mating type-associated incompatibility can in theory occur by mutations in either the mating type genes or *tol*. In the case of *N. sitophila* and *N. tetrasperma*, it was shown that the mating type idiomorphs display incompatibility when introgressed into *N. crassa* (58, 69), while introgression of tol^{+} from *N. crassa* into *N. tetrasperma* induces mating type-associated incompatibility (48). These results suggest that the absence of incompatibility in these species is due to *tol*.

What is the significance of mating type-associated incompatibility? As proposed for other *het* genes, the emergence of mating type-associated incompatibility might be accidental. This would explain why its occurrence is not systematic. Alternatively, it has been proposed that mating type-associated incompatibility might favor outbreeding by preventing the formation of mixed mating type heterokaryons between siblings of the same cross (83). If such a heterokaryon forms, it is likely that the subsequent cross will occur between the two nuclear types of the heterokaryon, thus leading to pseudohomothallism. If a single *het* locus were to favor outbreeding in that way, tight linkage to the mating type locus would ensure optimal efficiency.

het-c Is under Balancing Selection

The incompatibility reaction caused by heteroallelism at *het-c* is relatively mild compared to that of other *het* interactions in *N. crassa*. Forced heterokaryons or partial diploids heteroallelic for *het-c* display slow growth and are characterized by morphological alteration leading to a curly and aconiating mycelium (68, 79). Microscopic analyses indicate that about 15% of the cells are dead or dying in such partial diploids (49). Cell morphology and hyphal branching are affected, and cells appear swollen and round rather than cylindrical.

The $het\text{-}c^{OR}$ allele was cloned by locating crossover points between *het-c* and flanking markers in a cosmid spanning the region (79). This approach was combined with a functional transformation assay in which the selected cosmid clones were introduced into a recipient strain bearing the incompatible $het\text{-}c^{PA}$ allele to generate synthetic partial diploids that displayed the growth inhibition characteristic of *het-c* partial diploids. The *het-c* gene encodes a 966-amino-acid-long polypeptide with a putative signal peptide. The HET-c protein is characterized by a C-terminal glycine-rich repeated domain. This type of repeated domain was found in a number of extracellular proteins that, together with existence of the signal peptide, suggests that the HET-c polypeptide enters the secretory pathway. Inactivation of *het-c* by repeat-induced point mutation did not lead to any detectable phenotype. This could indicate that *het-c* does not play any cellular function in addition to its role in incompatibility or that the putative cellular function of *het-c* is redundantly mediated. A gene encoding a putative polypeptide of 798 amino acids displaying 27% identity and 28% similarity to $HET\text{-}C^{OR}$ was identified in the MIPS

Neurospora database (http://www.mips.biochem.mpg.de/proj/neurospora/). The polypeptide displays a signal peptide but lacks the glycine-rich domain.

At least three distinct alleles, designated *het-c*^{OR}, *het-c*^{PA}, and *het-c*^{GR}, exist at *het-c* (78). Overall, the three corresponding polypeptides display 86% identity, which is low for three allelic forms from the same species. The level of conservation varies in the different regions of the protein. In fact, three highly polymorphic regions can be distinguished, and outside of these regions conservation is 99%. Chimeric alleles show that the most polymorphic of these three regions controls allelic specificity. In a region 102 to 144 bp in length, the three alleles differ by numerous point mutations and two insertions: a 15-bp insertion found in the OR allele, and a 30-bp insertion found in the PA allele. Both insertions are absent in the GR allele. In that region, conservation between the three allele products is less than 25%. The three allelic types had equal frequencies of distribution, both in a field population of about 40 individuals and in a sample of 15 geographic isolates from various subtropical locations (96). The *het-c* locus was also analyzed in 12 other species in the family *Sordariaceae*. *het-c* was found to be polymorphic in all species analyzed. Strikingly, the three allelic types (OR, PA, and GR) defined by the specific insertion or deletion mutations could be identified in these species. The *het-c* polymorphisms are thus shared between species of the genus *Neurospora* and even between different genera in the *Sordariaceae*. The existence of this transpecific polymorphism indicates that polymorphism at *het-c* was present in an ancestral species. *Sordaria* and *Neurospora* diverged at least 36 million years ago; thus, the *het-c* polymorphisms have been maintained over an extraordinarily long period. Moreover, analysis of frequency of synonymous to nonsynonymous replacements showed that although there is a constraint to maintain the *het-c* sequence outside the specificity region, the specificity region is essentially unconstrained. Together, these results strongly suggest that balancing selection maintains polymorphism and allele frequencies at *het-c*.

Transpecific polymorphism is considered a strong criterion of positive Darwinian selection. The fact that *het-c* is under balancing selection suggests that this locus functions as a self/nonself recognition system. The *het-c* locus shares this property with known self/nonself recognition systems like the major histocompatibility complex class I and II loci and the S self-incompatibility locus in flowering plants (for a review, see reference 90). In these cases, transpecific polymorphism was evidenced for periods of up to 45 million years (36, 47, 67). Still, it remains possible that *het-c* polymorphisms have been maintained under neutrality if population sizes have remained very large throughout a very long period. An example of long maintenance (1.4 million years) of a transpecific polymorphism under neutrality has been reported in Lake Victoria cichlid fish (63).

The *het-c* homolog in *P. anserina* has been isolated and named *hch*. The *hch*-encoded polypeptide displays 52% identity with HET-C^{OR}. The *hch* gene was found not to be polymorphic in a sample of 11 wild-type isolates from France differing at all known *het* loci (76). Thus, the transpecific polymorphism is not seen in this species. This gene does not correspond to any of the known *het* loci of *P. anserina* and might not even function as a *het* locus in this species. This would date the emergence of *het-c* incompatibility before the divergence of *Neurospora* and *Sordaria* but after the divergence of *Neurospora* and *Podospora*, about 75 million years ago. *hch* differs from all three *N. crassa* allelic types in a region defining specificity and might thus represent an ancestral allele. Heterologous expression in *P. anserina* of the *het-c*^{PA} allele from *N. crassa* leads to a growth defect and a cell death reaction reminiscent of the one observed in partial diploids heteroallelic for *het-c* (76). This locus is therefore potentially active in incompatibility. In other words, if it were polymorphic, this gene could act as a *het* locus. In *Cryphonectria parasitica*, a number of incompatibility loci are not polymorphic or have extremely unbalanced allele distributions in certain populations (59). It therefore remains possible that *hch* is polymorphic and acts as a *het* locus in other *P. anserina* populations.

het-6 Region Contains Two Tightly Linked *het* Genes

Heteroallelism at *het-6* causes severe growth inhibition in partial diploids and heterokaryons. It was originally identified in partial diploids, but nearly isogenic strains differing at *het-6* have been constructed (86). The two incompatible haplotypes are designated *het-6*^{OR} and *het-6*^{PA}. The *het-6* locus was isolated by again using a combination of positional cloning and a transformation-based functional assay. *het-6*^{OR} activity was detected based on a reduction of transformation efficiency in an incompatible recipient strain. Surprisingly, two nonoverlapping fragments from the *het-6* region both display activity. Thus, at least two distinct incompatibility genes exist at that locus (85).

One of them is designated *het-6*^{OR}. It encodes a 680-amino-acid-long polypeptide (85). The alternate allele, *het-6*^{PA}, encodes a polypeptide that displays only 68% identity to HET-6^{OR}. This is the lowest identity level reported between alleles of a *het* locus. When the *het*-6^{PA} gene is introduced into a *het*-6^{OR} recipient strain, no reduction in transformation efficiency is detected. In other words, *het*-6^{PA} does not display PA incompatibility activity. It was therefore hypothesized that the *het*-6 gene in fact mediates incompatibility in a nonallelic fashion (85). In other words, incompatibility is not triggered by interaction of *het*-6^{OR} with *het-6*^{PA} but by the interaction of *het*-6^{OR} with a distinct gene. This nonallelic interaction must, however, involve a gene from the *het-6* region. Otherwise, progeny in which the two incompatible genes have been reunited by recombination in the same nucleus would be recovered in crosses between incompatible *het-6* haplotypes.

Smith et al. (85) identified a region of similarity between the HET-6 proteins and two other proteins involved in heterokaryon incompatibility, the HET-E protein and the *tol* product (81, 83). This region contains three conserved stretches that span a region of about 140 amino acids (Fig. 2). Otherwise, these proteins appear to be completely unrelated.

The second DNA fragment with OR incompatibility activity contains a gene encoding the large subunit of type I ribonucleotide reductase (84, 85). A temperature-sensitive lethal mutation (*un-24*) is strictly linked to *het*-6. *un-24* is a mutation in the gene encoding the large subunit of ribonucleotide reductase. It was proposed that the OR incompatibility activity of the second fragment is due to the ribonucleotide reductase-encoding gene. The *un-24*^{OR} and *un-24*^{PA} allele products differ greatly in the C-terminal end of the ribonucleotide reductase subunit in a region that is unique to the *N. crassa* ribonucleotide reductase-encoding gene. *un-24* complementation and OR incompatibility activity are separable (85). As for *het-6*^{PA}, the introduction of *un-24*^{PA} in an OR background does not lead to incompatibility. Although introduction of the *un-24*^{OR} allele into a PA recipient is lethal, the reciprocal transformation has no detectable effect. So, as for *het-6*^{OR}, *un-24*^{OR} appears to interact with a distinct but linked gene to trigger incompatibility.

The fact that *het-6* incompatibility is redundantly mediated by several genes is supported by the observation that escape from incompatibility in *het-6* partial diploids involves deletion

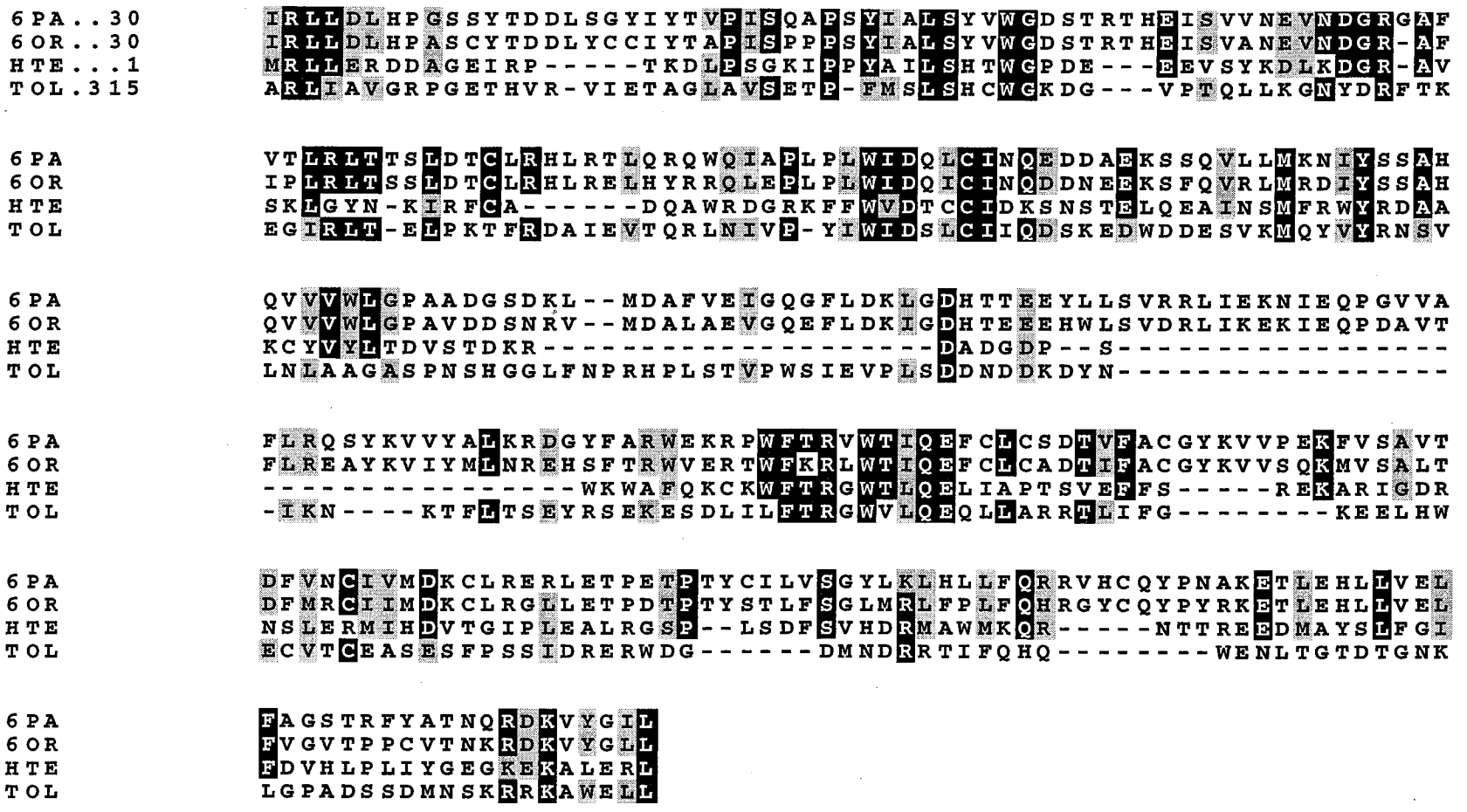

FIG. 2. Sequence motif common to several genes involved in nonallelic incompatibility. The amino acid position is given for each sequence: the *het-6*^{PA} allele product (6PA), the *het-6*^{OR} allele product (6OR), the *P. anserina* HET-E protein (HTE), and the *N. crassa* TOL protein (TOL). Residues that are identical in at least three of the four sequences are boxed in black; residues that are similar in at least three of the four sequences are boxed in grey.

of large chromosomal segments from the *het-6* region. Escape involves the deletion of either a 35-kb fragment of the OR haplotype or a 70-kb fragment of the PA haplotype (86).

Molecular characterization of the *het-6* locus, though incomplete, reveals an unexpected genetic complexity. Multiple genes are involved, and although they are genetically located in the same region, their interaction is apparently of the nonallelic type. *het-6*^{OR} and *un-24*^{OR} were always found associated, suggesting that recombination between these two genes is suppressed. Thus, the OR and PA specificities are defined as haplotypes corresponding to at least two different genes. The isolation of *het-6* has also revealed the existence of conserved regions common to three genes involved in incompatibility of the nonallelic type (85) (Fig. 2). It is tempting to consider this region of similarity a sort of "death domain" controlling common downstream targets. It is the first indication of a possible relationship between *het* genes in *N. crassa* and *P. anserina*.

HETEROKARYON INCOMPATIBILITY IN *PODOSPORA ANSERINA*

In *P. anserina* and many other species, incompatibility between two strains can be detected by the formation of an abnormal contact line termed the barrage (73). Thus, compatibility between strains can readily be determined by confronting the strains on solid medium. A genetic analysis of 17 wild-type isolates of *P. anserina* revealed the existence of at least nine *het* loci in that species (12). A battery of tester strains that are nearly isogenic except for a particular *het* locus were developed. The nine *het* loci define five allelic systems and three nonallelic systems; one locus (*het-V*) is simultaneously involved in an allelic and a nonallelic interaction. The genes belonging to one of the allelic systems (*het-s*/*het-S*) and to one nonallelic system (*het-c*/*het-e*) have been cloned (Table 1). In *P. anserina*, *het* gene isolation was achieved by functional cloning based on acquisition of a new incompatibility phenotype detected by a barrage reaction with a specific tester strain.

HET-s Protein as a Prion Analog

The *het-s* locus was described by G. Rizet in 1952 and was the first *het* locus to be thoroughly studied in *P. anserina* (73). Two incompatible alleles, *het-s* and *het-S*, exist at that locus. Confrontation of a *het-s* and a *het-S* strain leads to a barrage reaction. Interest in this locus was raised because of the cytoplasmic transmission of the [Het-s] character. When a cross is performed between a *het-s* and a *het-S* strain, 50% of [Het-S] strains are recovered in the progeny, but a proportion of the progeny of the *het-s* genotype fail to produce a "barrage" reaction with the [Het-S] parent (73). These strains define the neutral [Het-s*] phenotype. The proportion of [Het-s*] progeny is low if the female parent is [Het-s] but reaches 50% when the [Het-s] strain is the male parent in the cross (contributing very little cytoplasm). The maternal inheritance of [Het-s] is also illustrated by the fact that in a [Het-s] × [Het-s*] cross, all progeny have the phenotype of the female parent. [Het-s*] strains can be propagated vegetatively over extended periods of time but eventually spontaneously acquire the reactive [Het-s] phenotype. This phenotypic conversion can occur in any spot of the mycelium and then propagates as an infectious process throughout the entire mycelium at a speed of up to 70 mm a day (i.e., 10-fold faster than the growth rate). This conversion is also systematically induced after a cytoplasmic contact (anastomosis) with a [Het-s] strain. Conversely, a [Het-s] strain can return to the [Het-s*] phenotype when protoplasts are generated from the [Het-s] strain (10) or after fragmentation of the mycelium (9). In this case, a proportion of the strains that are generated from protoplasts or mycelial fragments display the neutral [Het-s*]. These thorough early genetic and physiological studies on the cytoplasmic inheritance of [Het-s] lead to the conclusion that "the presence of the cytoplasmic *s* factor is required for maintenance of its own synthesis" (9). In this early hypothesis, the bistability in the [Het-s] system is due to the existence of a regulatory loop reminiscent of the classical lactose permease induction system (65).

The molecular cloning of the *het-s* locus was possible because of the existence of a wild-type strain containing a natural null allele of *het-s* (29, 92). Using this strain as a recipient, a *het-s*-containing cosmid was isolated in a functional assay based on the acquisition of the barrage reaction with a *het-S* strain. The *het-s* and *het-S* alleles encode two highly homologous proteins of 289 amino acids in length that differ at 13 amino acid positions (91). In the *het-s* and *het-S* ORFs, of 17 substitutions, only 3 are synonymous. The ratio of synonymous to nonsynonymous substitutions is similar to that of a pseudogene. No intraspecific variability was found within an allelic type (29). All wild-type sequenced *het-s* alleles are identical, and all wild-type sequenced *het-S* alleles are identical. This could indicate that polymorphism at *het-s* is ancient and has been generated in an ancestral species. Analyses of the *het-s* sequences from natural isolates revealed that all *het-s* strains contain a 354-bp-long transposon long terminal repeat inserted in the promoter sequence and a 2-kbp insertion downstream of the ORF (30). It is therefore likely that recombination at this locus is suppressed.

Disruption of these genes does not cause any growth defect or detectable phenotype other than the loss of the *het-s*-based incompatibility. Genetic dissection of *het-s* was undertaken using various approaches, including construction of chimeric alleles, site-directed mutagenesis, and a genetic screen based on the selection of mutants that escape self-incompatibility of a strain coexpressing *het-s* and *het-S* (25, 29). A single-amino-acid substitution is sufficient to switch to the antagonistic specificity. The HET-S protein has a histidine at amino acid position 33. Replacement of this amino acid by a proline (as found in HET-s) gives rise to a protein of the HET-s specificity (29). Numerous other amino acid replacements or even deletion of this amino acid gives rise to a protein of the [Het-s] specificity. Single-amino-acid replacements at other positions in the N terminus (positions 25 and 38) lead to a specificity switch from [Het-S] to [Het-s] (25). All of these mutant alleles have all the properties of the wild-type *het-s* allele, i.e., a strain containing this allele can express the alternate [Het-s*] and [Het-s] phenotypes. Together, these results indicate that *het-s* specificity is defined by the N terminus of the protein. Also, several single-amino-acid substitutions in that region lead to a specificity switch. Incompatibility can thus be triggered by coexpression of two proteins that differ by only one amino acid.

When antibodies to HET-s became available, it was determined that the *het-s*-encoded protein is present in both [Het-s*] and [Het-s] strains. Therefore, it is not the absence of the *het-s*-encoded protein that accounts for the nonreactive [HET-s*] phenotype. Thus, the regulatory loop model had to be rejected. What are the alternative models? Could the [Het-s] element be a cytoplasmic nucleic acid requiring the nuclear *het-s* gene to be propagated? The hypothesis is unlikely because [Het-s*] strains can be recovered from [Het-s] strains (for example, when protoplasts are produced, or in the progeny of a cross with *het-S*). These strains can then spontaneously reacquire the [Het-s] phenotype. If [Het-s] is a nucleic acid replicon, it is difficult to devise a simple model explaining how, once lost, this putative replicon can appear de novo. Coustou

et al. proposed that the [Het-s] cytoplasmic element is a prion (24). Prions are infectious proteins. The term prion was coined to define the infectious agent of spongiform encephalopathies. Transmission of this infectious agent occurs as a transmissible conformational modification in a cellular protein called Prp (for a review, see reference 72). This type of protein-based inheritance is not restricted to Prp. Two non-Mendelian elements of *Saccharomyces cerevisiae*, the [URE3] and [PSI] elements, are in fact prions (for a review, see reference 95). Identification of these two long-known non-Mendelian genetic elements as yeast prions was first based on genetic criteria that distinguish them from nucleic acids (94). Biochemical analysis then confirmed that these elements propagate by self-aggregation of the Ure2p and Sup35p proteins, respectively. In the prion model, in [Het-s*] strains, the protein exists in a nonreactive conformation, HET-s*. In [Het-s] strains, the protein is in the reactive HET-s conformation, and HET-s can catalyze the conversion of HET-s* into HET-s. This model readily explains the unusual genetic and physiological properties of the [HET-s] element. Namely, it accounts for the cytoplasmic inheritance of the [Het-s] element, the rapid and infectious transmission of [Het-s], and the reversible curing of [Het-s] (loss of the [Het-s] character and spontaneous reappearance). The [Het-s] system displays additional properties that are common to all prion systems. First, as expected in an autocatalytic system, overexpression of the *het-s*-encoded protein increases the frequency of spontaneous appearance of the [Het-s] phenotype. Second, the HET-s protein is more resistant to proteinase K digestion than HET-s*, which indicates the occurrence of some sort of modification of the protein.

A genetic screen based on the escape from *het-s*/*het-S* self-incompatibility confirmed the role of the N-terminal part in defining the prion properties (25) and showed that the incompatibility function and the prion-inducing function can be separated. A *het-s* mutant with a premature stop codon at position 26 was obtained. This strain has lost the incompatibility function but is capable of converting a [Het-s*] strain to the [Het-s] phenotype. Thus, a short N-terminal peptide from HET-s is sufficient to propagate [Het-s].

Preliminary biochemical and cytological analyses (using green fluorescent protein fusion proteins) indicate that, as for the other prion systems, transition to the prion form renders the HET-s protein prone to aggregation (V. Coustou and S. Saupe, unpublished results). Moreover, when expressed in *Escherichia coli*, the HET-s and HET-S proteins display very different solubility properties. In conditions that allow expression of soluble HET-S, the HET-s protein is highly insoluble. These differences in solubility are determined by the same amino acid position that defines allele specificity in incompatibility (V. Coustou and S. Saupe, unpublished results).

The puzzling genetic behavior of [Het-s] has received a satisfactory explanation with the prion hypothesis, but how the interaction of the two antagonistic proteins controls cell death remains completely unknown. It has been shown that HET-S and HET-s are able to interact in a yeast two-hybrid system (24). It will be interesting to see whether there is a connection between the prion behavior of HET-s and the incompatibility reaction. The cell death reaction might for instance be caused by the coaggregation of the HET-S protein with HET-s. It has been proposed that in the case of [PSI], aggregation of Sup35p into higher-order aggregates actually protects the cells from the toxic effect of misfolded Sup35p (20). Based on this hypothesis, one might propose that incorporation of HET-S into HET-s aggregates inhibits further aggregation of HET-s and thus causes accumulation of misfolded HET-s, which has a toxic effect.

For the two yeast prions, the presence of the prion has the same phenotype as the null mutation of the gene that is required for prion propagation. Transition to the prion form leads to a loss of function of the protein. The opposite is true in the case of [Het-s]. Here the prion is the reactive form of the protein, the form that specifically interacts with HET-S to trigger the incompatibility reaction. This has important implications. First, it indicates that acquisition of the [Het-s] phenotype cannot be explained solely by the aggregation of the protein leading to a loss of function. Then, it suggests that a prion need not necessarily be a "pathological" alteration of a protein, a notion that is also supported by the fact that the presence of the yeast [PSI] prion can be beneficial under certain stress conditions (32). This conclusion is only valid, however, if one assumes that the only biological function of *het-s* is to control heterokaryon incompatibility. It remains possible that an as yet unknown cellular function of the HET-s protein is lost during the transition to the prion state, as for the yeast prions. The existence of a null allele of *het-s* in a wild-type strain with a premature stop codon argues against that. Conversely, if *het-s* functions only in incompatibility, the emergence of a null allele is possible in a population that contains only one allele because in this case no constraint exists to maintain *het-s*.

What would be a possible scenario for emergence of the incompatibility function at *het-s*? Numerous single-amino-acid substitutions can switch a HET-S protein to the [Het-s] specificity. Thus, the emergence of the incompatibility function is relatively likely to occur, as it requires only a minor mutational event. Then, if a selective advantage is associated with the incompatibility function, *het-s* allele frequency can increase in the population. There is a phenomenon that could have increased *het-s* allele frequency even in the absence of selection for the heterokaryon incompatibility function. The *het-s* allele behaves as a spore killer (12). At 18°C in a *het-s* × *het-S* cross in which *het-s* is the maternal parent, in a proportion of the asci, the *het-S* spores are abortive. In these asci, the spores of the *het-s* genotype display the [Het-s] phenotype upon germination, whereas in the normal four spored asci, the spores of the *het-s* genotype display the [Het-s*] phenotype upon germination. There is thus a correlation between killing activity and expression of the reactive [Het-s] phenotype. Similarly, [Het-s*] strains or strains in which *het-s* is disrupted fail to kill. Such *het-s* × *het-S* crosses produce an excess of *het-s* offspring over *het-S* offspring. These characteristics tend to support the "accident" hypothesis, as both emergence of the incompatibility function and allele fixation can be explained in the absence of selection for the incompatibility function.

Nonallelic Incompatibility

In nonallelic incompatibility systems, cell lysis is triggered by the interaction of two unlinked genes. Three allelic systems have been described in *P. anserina* (*het-c*/*het-d*, *het-c*/*het-e*, and *het-r*/*het-v*). Because the alleles causing incompatibility are unlinked, they can be reunited in the same nucleus, generating self-incompatible progeny. Such self-incompatible spores germinate normally, but after about 10 hours, growth stops and cells undergo a generalized lysis reaction characterized by intense vacuolization and ultimately complete degeneration (Fig. 3). These self-incompatible strains have proven to be powerful tools to select for suppressors of incompatibility and to analyze the biochemical modification that characterizes the incompatibility reaction.

Cell lysis is associated with changes in gene expression. Numerous catabolic enzymatic activities (laccases, phenoloxi-

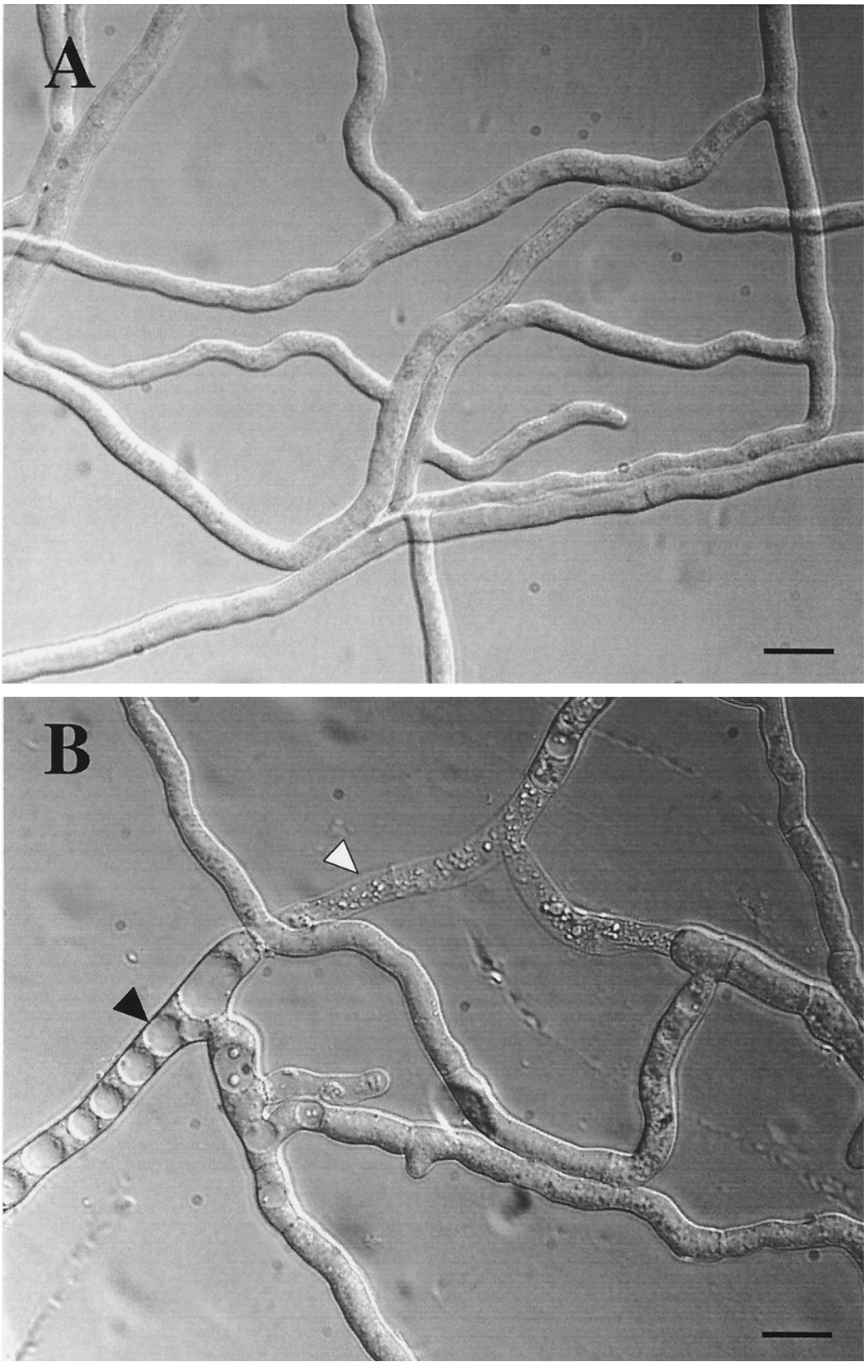

FIG. 3. Cell death reaction occurring in a self-incompatible *het-C het-E* strain of *P. anserina*. A *het-c het-e* strain is shown in panel A (non-self-incompatible control), and a *het-C het-E* self-incompatible strain is shown in panel B. The mycelium was photographed 12 h after spore germination. Bar, 10 μm. Note the intense vacuolization (black arrowhead) and the presence of completely destroyed cells (white arrowhead).

dases, and proteases) are induced (16). The *het-r*/*het-v* system is particularly suited for these studies because the *het-R*/*het-V* strain cell lysis reaction is conditional. A *het-R het-V* strain shows normal growth at 32°C, but shifting the growth temperature from 32 to 26°C induces a generalized cell lysis reaction. All three nonallelic systems have common suppressors, mutations in the *mod-A* and *mod-B* genes. It is therefore likely that all nonallelic *het* genes are involved in the same biological pathway.

The nonallelic systems in *P. anserina* have an additional property that distinguishes them from allelic systems. They behave as sexual incompatibility systems. A cross between incompatible strains can be partially or even totally sterile. Fertilization fails presumably because the incompatibility reaction occurs when the gametes meet. It has been proposed that due to this partial reproductive isolation, *het* genes might participate in speciation (12).

Current studies on nonallelic incompatibility are based on three complementary approaches: characterization of the *het* genes, of the suppressor genes, and of the genes that are induced during the incompatibility reaction (Tables 1 and 2).

***het-c* and *het-e* genes.** The *het-c* and *het-e* loci are both multiallelic. Four *het-c*, four *het-e*, and three *het-d* alleles have been described in wild-type isolates (12). Each *het-c* allele is incompatible with a specific set of *het-e* and *het-d* alleles. The *het-c2* allele has been cloned using a functional assay based on acquisition of the barrage reaction with a *het-e1* strain (77). It encodes a 208-amino-acid protein which displays similarity to a glycolipid transfer protein (GLTP) from pig brain (1). Orthologs of this gene also exist in humans and species of the genera *Arabidopsis* and *Caenorhabditis*. In the last two species, two different GLTP-like genes were found, suggesting the possible existence of a gene family. Inactivation of *het-c* in *P. anserina* does not affect vegetative growth, but in a cross homozygous for the *het-c* deletion, ascospore maturation is drastically impaired (77). Thus, *het-c* has a cellular function. Therefore, like the mating type genes and *un-24*, *het-c* is not involved solely in heterokaryon incompatibility. The exact cellular function of the GLTPs is not known. In vitro, the pig brain GLTP binds several glycosphingolipids and glyceroglycolipids and allows their transfer between donor and acceptor liposomes (1, 74). It has been proposed that transfer of glycolipids from the endoplasmic reticulum to the Golgi relies on a nonvesicular transport system in which these proteins might be involved. Alternatively, it is possible that the transfer activity measured in vitro does not actually represent a physiological activity; these proteins might simply bind glycolipids without actually translocating them. At this time, it is not possible to explain how disruption of the proposed glycolipid transfer (or binding) activity of HET-c affects ascospore maturation.

The sequences of the three additional wild-type *het-c* alleles have been determined (82). The four allelic forms are 92% identical. A region of high variability (positions 126 to 160) can be identified in which identity drops to 77%. Mutant alleles of *het-c* have been selected by screening for restoration of fertility with a strain of the incompatible *het-e* and *het-d* genotype (7). Two such mutant alleles displaying an altered specificity have been sequenced. Two key residues for specificity have been identified (amino acid positions 133 and 153). These mutations selected for alteration of *het-c* specificity correspond to polymorphic positions in the four wild-type isolates. They correspond to nonconserved positions in the different GLTP-like proteins. Sequence comparison between the two most divergent alleles, *het-c2* and *het-c3*, revealed an excess of nonsynonymous substitutions over synonymous substitutions (15 of 17 substitutions are nonsynonymous). Moreover, the frequency of substitutions is more than twofold higher in the coding region (ORF) than in the noncoding regions (5′ and 3′ regions and introns) (82). Conservation of *het-c*-related genes in very distant species together with the phenotype conferred by the *het-c* inactivation indicates that a constraint exists to maintain a functional *het-c* sequence. Thus, the high occurrence of nonsynonymous substitutions cannot be due to lack of purifying selection. This suggests that *het-c* is evolving fast and that polymorphism at certain amino acid positions modifying specificity might be selected for, while the putative glycolipid transfer activity is maintained.

One allele of *het-e* was also cloned using the same functional approach that was used to clone *het-s* and *het-c*. It encodes a 1,056-amino-acid polypeptide. The N-terminal region comprises the region of similarity with TOL and HET-6, followed by a GTP binding site (P-loop motif) and a C-terminal WD repeat domain (81). The HET-e protein binds GTP in vitro, and mutations that abolish binding also abolish activity in incompatibility (33, 81). The WD repeat domain was first described in the β subunits of trimeric G proteins. It is composed of a variable number of repeats of about 40 amino acids in length. It is a very common structure found in a large number of other proteins (at least 55 in *S. cerevisiae* alone) (for a review, see reference 89). The structure of the Gβ subunit, the prototypical WD repeat protein, is known. It adopts a β-propeller fold, a circular structure composed of modules of four-stranded antiparallel β-sheets organized around a central pore. This structure has two large flat surfaces that are believed to provide interaction sites for other protein partners (87). The HET-e1 protein displays 10 WD repeats. Allele specificity is not defined by the number of repeats, but alleles displaying fewer than 10 repeats are inactive in incompatibility, which suggests that a minimal number of repeats are required for incompatibility (33, 81). For the other WD repeat proteins, it was suggested that binding specificity was determined by the surface residues of the flat surface of the β-propeller structure (87). It will be interesting to see if *het-e* specificity is determined by these surface residues.

Inactivation of *het-e* by gene replacement has no detectable effect during vegetative growth or during the sexual phase. In particular, the defect in ascospore production seen in *het-c* mutants was not observed. Sequences homologous to *het-e* exist in the *P. anserina* genome (33, 75). It is therefore likely that paralogs of *het-e* exist and complement its inactivation. Based on its genetic interaction with *het-c*, *het-d* is one obvious candidate for such a paralog.

How do HET-e and HET-c interact to trigger incompatibility? The GTP-binding activity of HET-e suggests that it might have a role in signal transduction. Glycolipids, in particular ceramides, have been implicated in a variety of cellular signaling systems as mediators of stress response and both apoptotic and nonapoptotic cell death reactions (for a review, see reference 57). HET-c and HET-e might be components of a signaling system, and an incompatible interaction might then correspond to a constitutive activation of the signaling pathway.

Of all the WD repeat proteins, HET-e is the only one for which very strong conservation between repeats has been described (conservation between repeats ranges from 81 to 98%). This conservation is correlated with the existence of VNTR (variable number of tandem repeats) polymorphism. The number of WD repeats varies between alleles. It ranges from 3 to 10 in wild-type *het-e* alleles (33, 81). VNTR polymorphism can be generated by unequal crossovers which lead to expansion or reduction of the number of repeats. As a consequence, these repeated sequences are subject to concerted evolution. A randomly sampled repeat variant can invade the whole array of

repeats. This rapidly generates extensive polymorphism. It has been proposed that "genes evolving by concerted evolution produce a selective force on the gene of their cognate interacting protein" (8). This requires no external forces. For example, the rapid evolution of the lysin sperm protein of abalone, a marine mollusk, is driven by concerted evolution in the tandemly repeated vitelline envelope receptor for lysin. Repeat comparison in the *het-e* allele and the existence of VNTR polymorphism suggest that *het-e* is subject to concerted evolution (33, 81). It can therefore be hypothesized that the proposed rapid evolution of *het-c* is driven by the occurrence of concerted evolution in *het-e*. In other words, rapidly emerging *het-e* variants could select for adapted variants of *het-c*. Incompatibility would result from the interaction of a *het-e* allele with an unadapted *het-c* allele. In this hypothesis, one does not need to invoke positive selection on the incompatibility function to explain the rapid evolution of *het-c* alleles.

***mod-A* suppressor and other *mod* genes.** Suppressors of nonallelic incompatibility can be selected in haploid self-incompatible strains in which two unlinked incompatible genes are reunited in the same nucleus. Self-incompatible spores of this genotype stop growing a few hours after spore germination, and then a generalized lytic reaction occurs in the thallus. The *mod-A1* mutation prevents the growth arrest due to the incompatible *het* gene interaction (11). It is recessive and does not lead to complete suppression of the incompatibility reaction as *tol* does in *N. crassa*. A *het-C het-E mod-A1* strain displays continuous growth, but the characteristic lytic reaction occurs in the older parts of the thallus. To allow complete suppression of incompatibility, the *mod-A1* mutation must be associated with a second mutation in the unlinked *mod-B* locus. In the *mod-A1 mod-B1* double mutant, all three nonallelic incompatibility interactions are suppressed (13, 51). The *mod-A1 mod-B1* double mutant displays developmental defects in a compatible genetic context. This strain is female sterile. It has a defect in the development of the female reproductive organ, the protoperithecium (13, 15). The female sterility of *mod-A1 mod-B1* strains is conditional and can be suppressed by addition of certain amino acids, such as phenylalanine, asparagine, tyrosine, and leucine, to the culture medium (17). It is also dependent on the alleles present at the nonallelic *het* loci. For example, certain mutations in *het-e* and *het-d* abolish the residual fertility of *mod-A1 mod-B1* strains. These results were the first indication that a link exists between the development of female reproductive structures and nonallelic incompatibility. The *mod-A* gene was isolated by identifying a cosmid restoring the female fertility of a *mod-A1 mod-B1* strain (5). It encodes a 687-amino-acid polypeptide. The C-terminal part of the polypeptide contains a proline-rich motif that displays some similarity to an SH3-binding domain.

A specific suppressor of the *het-R het-V* interaction has also been described (52). The *mod-C* mutations specifically suppress *het-R het-V* incompatibility but have no effect on *het-C het-D* or *het-C het-E* interactions. This indicates that the cell death pathways induced by the *het-R het-V* and *het-C het-E* interactions are at least partially distinct. Similar to *mod-A1 mod-B1* double mutants, the *mod-C1* mutant is female sterile.

A number of additional *mod* genes (*mod-D* through *mod-G*; for a review, see reference 31) have been described. These other *mod* mutations have been selected in complex genetic contexts but not as direct suppressors of incompatibility. For example, *mod-E* is a suppressor of some of the developmental defects of a *mod-D* mutant, which in turn restores growth of a *het-C het-E mod-A1 mod-C1* double mutant. Therefore, their direct connection with the incompatibility reaction is not always obvious. Both MOD-E and MOD-D are proposed components of signal transduction pathways involved in the control of developmental processes (56). The *mod-D* gene encodes an α subunit of a trimeric G protein (56). At low temperature, the radial growth of a *het-C het-E mod-A1* strain is slightly increased by mutations in *mod-D. mod-D* mutations have no suppressive effect on incompatibility in a wild-type background. The *mod-E* gene encodes an HSP90 (55). A partial suppression of the *het-R het-V* incompatibility by mutations in a *mod-E* strain has been reported. It has been suggested that these results further strengthen the hypothesis of a connection between developmental pathways and incompatibility (56). However, since both *mod-E* and *mod-D* encode components of essential signal transduction pathways, it is possible that the described partial suppressive effect on incompatibility is indirect.

Genes induced during the incompatibility reaction. When the incompatibility reaction is induced in a *het-R het-V* strain, at least a dozen new polypeptides appear (18). The cloning of these induced genes was undertaken by a differential hybridization approach. Three *idi* genes (for induced during incompatibility) have been characterized to date. All three genes (*idi-1*, *-2*, and *-3*) encode small proteins with signal peptides and are characterized by a very high expression level during incompatibility (each one represents about 1% of the mRNA). The IDI-2 protein is cysteine rich and displays a region of similarity with IDI-3. Induction of all three *idi* genes is diminished in a *het-R het-V mod-C1* background. *idi-1* and *idi-3* are also induced in *het-C het-E* self-incompatible strains but not by the *het-s*/*het-S* interaction. Moreover, their induction is abolished in a strain bearing the *mod-A1 mod-B1* mutations. In contrast, *idi-2* is only induced by the *het-R het-V* interaction, and its expression is not affected in a *mod-A1 mod-B1* double mutant. This again illustrates the fact that the cell death pathways are partially distinct in the *het-R het-V* and *het-C het-E* systems.

Inactivation of the *idi* genes will indicate if these genes are directly responsible for the cell lysis reaction.

It has been shown that development of the incompatibility reaction leads to the appearance of a specific proteolytic activity (7). A protease specifically induced when a *het-R het-V* strain is shifted from 32 to 26°C has recently been purified. The corresponding gene encodes a 532-amino-acid serine protease precursor of the subtilisin type (M. Paoletti and C. Clavé, unpublished results). Subtilisins are broad-specificity endopeptidases with an S-H-D catalytic triad. *pspA* shows strong similarity to the *pepC* protease from *Aspergillus niger* (37) and the *prB* protease from *S. cerevisiae* (60). Both are proposed vacuolar proteases and are distinct in sequence from secreted proteases. This, together with the fact that *pspA* was purified as an intracellular enzyme, suggests that it might be vacuolar (M. Paoletti and C. Clavé, unpublished results). *pspA* expression is induced in a *het-R het-V* strain and also in a *het-C het-E* strain. Disruption of *pspA* does not prevent development of the incompatibility reaction. This indicates that additional lytic activities contribute to the cell death reaction.

Other physiological conditions induce this protease, nitrogen or carbon starvation and exposure to light. Moreover, inactivation of *pspA* by gene disruption leads to pleiotropic growth defects (M. Paoletti and C. Clavé, unpublished results). Vegetative growth is affected, as no aerial structures are produced. This mutant strain is female sterile. It differentiates much fewer female reproductive structures (protoperithecia), and these protoperithecia fail to mature after fertilization. Finally, spore germination is also affected. Thus, *pspA* is clearly not involved solely in the incompatibility reaction but also participates in various differentiation steps.

Comments. In *P. anserina*, development of female structures (protoperithecia) occurs once the culture medium is exhausted and is accompanied by the cell death of surrounding vegetative hyphae (14). This autolysis presumably provides the nutrients required for differentiation of the female reproductive structures. As no asexual spores are produced in *P. anserina*, production of sexual spores is the only way to escape adverse conditions in this species. It has been suggested that the *mod-A* and *mod-B* genes together with the nonallelic *het* genes control this limited self-lysis process. This model was in particular based on the fact that *mod-A1 mod-B1* mutants are defective for protoperithecium and perithecium production and that this sterility can be suppressed by addition of amino acids to the culture medium or by certain allele combinations of the nonallelic *het* loci (17). Recent data show that the *pspA* protease induced during the incompatibility reaction is also required for differentiation of female structures and induced by carbon and nitrogen starvation. The gene encoding another protease, the *papA* aspartyl protease, is also induced by carbon starvation (66). This latter protease is not involved in incompatibility, but its induction is abolished in a *mod-A1* mutant. This again suggests that the *mod-A* gene could be part of a starvation response pathway. These molecular results support the notion that the pathway that leads to cell death in nonallelic incompatibility employs components of a pathway that controls adaptation to starvation and subsequent development of female reproductive structures.

How do *het-c* and *het-e* fit into this model? These *het* genes might be part of the normal differentiation pathway as possible sensors or transducers of some environmental signal. Alternatively, it is possible that an incompatible *het-c*/*het-e* interaction affects cell metabolism in a way that is perceived as a differentiation signal leading to induction of the self-lysis pathway.

GENERAL CONCLUSIONS AND FUTURE PROSPECTS

What have we learned from the molecular analyses of the *het* genes and of the other genes involved in incompatibility? These analyses have revealed a great diversity in the genetic mechanisms leading to incompatibility. The incompatible genes can be true alleles of the same locus (*het-s*/*het-S*), idiomorphs (mating type genes), two alleles from unlinked genes (*het-c*/*het-e*), or distinct but tightly linked genes (*het-6*/*un-24* region). The different *het* genes encode different, nonhomologous proteins. They obviously do not belong to a single gene family. A number of common characteristics have become evident even if they are not systematically shared by all known *het* genes. First, there is generally a high variability between allelic forms. Even if experimentally very limited allelic differences are sufficient to lead to incompatibility, the incompatible alleles generally differ greatly. In a number of cases, there appears to be extensive divergence in the regions that surround the *het* genes and a local suppression of recombination. For example, the sequences upstream and downstream of the *het-s* and *het-S* alleles in *P. anserina* differ by the presence of a transposon long terminal repeat and a 2-kbp deletion (30, 92). The sequences downstream of the *het-c* ORF differ totally in *het-c*^{PA} and *het-c*^{OR} (S. Saupe and L. Glass, unpublished data), and recombination at the *het-6* locus appears to be suppressed (85). This possible suppression of recombination due to apparent rearrangements could promote accumulation of extensive variability in the coding sequences of the *het* genes. The high intraspecific divergence in the coding regions of *het* genes suggests that these genes are subject to rapid evolution. In a number of cases, there are indications that positive selection might be operating to promote polymorphism at these loci. The most convincing example is certainly the *het-c* locus of *Neurospora* because transpecific polymorphism can be evidenced, but the high variability found in other loci could also support this view.

As discussed in the Introduction, the detection of positive selection favors the allorecognition hypothesis. Milgroom and Cortesi analyzed the allele frequency for six *het* loci in 13 natural *C. parasitica* populations (59). In most cases, allele frequency deviated from the equilibrium frequency, thus providing no evidence for positive selection acting on these *het* loci. The results of this global survey therefore do not support the allorecognition hypothesis. Similarly, the work on nonallelic incompatibility in *P. anserina* convincingly illustrates the accident hypothesis. The *het-c* gene has a cellular function. The downstream cell death pathway is not specific to incompatibility but apparently corresponds to an autolysis pathway induced by starvation and involved in female structure development. Moreover, one does not need to invoke positive selection to account for the appearance of polymorphism in the *het* genes, since rapid evolution of *het-c* might well be driven by concerted evolution in the tandem repeats of *het-e*. It might be a mistake to look for a global hypothesis explaining the emergence of all incompatibility systems. It is conceivable that both the accident and the allorecognition hypotheses are valid depending on the species or *het* locus that is considered.

What do we need to know now? First, concerning the problem of the biological significance of incompatibility, more analyses on *het* gene variability in natural populations are needed to determine if positive selection is indeed a force driving the evolution of these genes. Then, one of the central questions regarding incompatibility remains nearly totally unanswered. The mechanism by which *het* gene interactions lead to cell death is unknown. It is likely that this involves direct protein-protein interactions between the incompatible products, but that is about all that is known. It is now important to establish the precise cellular function of a *het* gene to understand how perturbation of this function kills the cells. The *un-24* gene of the *het-6* region in *N. crassa* might be particularly well suited to such studies. There is an essential and well-documented enzymatic activity associated with the *un-24* gene product (84, 85). This should enable a direct analysis of the perturbation occurring during the incompatibility reaction. Dissection of the cell death pathway in *P. anserina* provides access to the genes controlling the cell death reaction. The identification of a common sequence motif in three genes involved in nonallelic incompatibility suggests that it could correspond to a sort of death domain controlling common downstream effectors. It will be of great interest to see if the homologs of the *idi* and *pspA* genes characterized in *P. anserina* are also induced by *Neurospora het* systems.

Heterokaryon incompatibility is an intriguing phenomenon both at the evolutionary level and in terms of mechanisms of molecular recognition. A decade of work on the molecular genetics of heterokaryon incompatibility left us with numerous pieces of the jigsaw puzzle. Hopefully, the next decade will allow their assembly into an informative mosaic. A general approach in biological investigations is to try to understand a system by perturbing it. Vegetative incompatibility is a naturally occurring perturbation of the cell leading to death. Study of this phenomenon constitutes an original problem in fungal biology, one which will certainly continue to provide original insights into various cellular processes.

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